Volume 4 Number 6 June 1977 Nucleic Acids Research

Comparative oligonucleotide fingerprints of three plant viroids

Hans J. Gross, Horst Domdey, and Heinz L. Sänger+

Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, and +Arbeitsgruppe Pflanzenvirologie, Justus Liebig-Universität, D-6300 Giessen, GFR

Received 29 March 1977

ABSTRACT
5' Phosphorylation in vitro with γ-^{32}P-ATP and T4 phage induced polynucleotide kinase was used to obtain RNAase A and RNAase T_1 fingerprints of three plant viroids: Potato spindle tuber viroid from tomato (PSTV-tom), chrysanthemum stunt viroid from cineraria (ChSV-cin) and citrus exocortis viroid from Gynura aurantiaca (CEV-gyn). These three viroids differ significantly from each other as judged from their oligonucleotide patterns. This supports the concept of individual viroid species.

INTRODUCTION

Since 1971 several groups have characterized pathogens of higher plants, which formerly were regarded as viruses albeit they seemed to be untractable with the methods of classical virology. These new infectious agents, called viroids, were found to be small replicating, infectious and pathogenic ribonucleic acid molecules not ensheathed in a protein coat like conventional viral RNAs (1).

Recently, we have shown that viroids are single-stranded, covalently closed circular RNA molecules with molecular weights of 115 000 to 127 000 daltons, existing in their native state as highly basepaired rod-like structures (2,3). In this report we present comparative oligonucleotide fingerprints of three different viroid isolates, using T4 induced polynucleotide kinase for 5'phosphorylation with γ-^{32}P-ATP in vitro.

MATERIALS and METHODS

General: RNAase T_1 (E.C.3.1.4.8), RNAase A (E.C.3.1.4.22), alkaline phosphatase from E.coli (E.C.3.1.3.1), and yeast hexokinase (E.C.2.7.1.1) were from Boehringer Mannheim GmbH. Precoated thinlayer chromatography plates for homochromatography (CEL 300 DEAE/HR-2/15,20x40 cm) were bought from Macherey, Na-

gel u. Co., Düren; cellulose acetate strips (3 x 55 cm) were from Schleicher and Schüll, Dassel.

Solutions for homochromatography were prepared as described by Jay et al. (4), except that the pH of the final "50 mM homomix" was adjusted to 4.7 with acetic acid (5). Kodak RP Royal X-Omat RP/R-54 X-ray film was used for autoradiography.

Origin and propagation of viroids: The Californian isolate of CEV was kindly supplied by Dr. L.G. Weathers, Riverside, in Etrog citron (Citrus medica) from which it had been adapted to the botanically unrelated herbaceous composite Gynura aurantiaca DC (6,7). The isolate of PSTV, a gift from Dr. T.O. Diener in the tomato (Lycopersicum esculentum) cultivar "Rutgers" (8), was propagated in the bush tomato "Rentita". Dr. M. Hollings, Littlehampton, kindly provided the isolate of ChSV in the Chrysanthemum morifolium cultivar "Mistletoe" (9) from which it was adapted to and propagated in the botanically closely related florists cineraria (Cineraria hybrida) cultivar "Hansa" (10).

Successful viroid infection and replication was indicated by the characteristic symptoms of stunting and epinasty in the host plants (7). Moreover, the appearance of the extra band of viroid RNA not present in the RNA preparations from healthy control plants was ascertained in stained polyacrylamide gels as shown previously (2).

Isolation of viroids: Viroids were purified from total nucleic acids extracted from systemically infected leaf tissue by a combination of conventional methods including fractionation with NaCl and cetyltrimethylammonium bromide followed by three preparative polyacrylamide electrophoreses (2,11).

In vitro ^{32}P labeling and fingerprinting of viroid RNA: γ-^{32}P-ATP was prepared as described by Glynn and Chappell (12); the freshly synthesized material had a specific activity of about 100 Ci/mmole. Polynucleotide kinase was prepared from T4 phage infected E.coli by extraction of the cells, streptomycin precipitation, autolysis, ammonium sulfate fractionation, DEAE-cellulose, phosphocellulose, and hydroxyapatite fractionation as described by Richardson (13), followed by a pH-gradient elution from a phosphocellulose column (14). The final enzyme fractions were dialyzed against 25 mM KCl - 10 mM mercaptoethanol - 20 mM

potassium phosphate pH 7.0 in 50 % glycerol, and stored at -20° C. 5 µg of viroid RNA were digested with 0.6 units RNAase T_1 or 0.5 µg pancreatic RNAase in 17 µl 60 mM Tris-HCl, pH 8.0 for 3 h at 37° C. This digest was treated with 0.002 units of bacterial alkaline phosphatase (2 h, 37° C); the phosphatase was then inactivated by nitrilotriacetic acid treatment as described by Simsek et al. (15). From this digest (20 µl), 5 µl were used for in vitro 5'end group labeling of the fragments produced by RNAase T_1 or pancreatic RNAase digestion. 5'Labeling was performed according to Simsek et al. (15). Twodimensional high voltage electrophoresis/homochromatography: An aliquot of the 5' ^{32}P labeled RNA digest was fractionated by electrophoresis (5000 V) on cellulose acetate in 7 M urea-pyridine acetate pH 3.5 (16), whereby the blue marker dye xylene cyanol FF was allowed to move 8 cm. The transfer of the material from cellulose acetate (first dimension) to DEAE thinlayer plates (second dimension) was performed as described by Southern (17). The plates were rinsed with ethanol to remove urea, and dried at room temperature. Homochromatography was carried out at 65° C as follows: The plate was equipped with a wick and first put in water, which was allowed to run 10 cm. The wet plate was then transfered into a "50 mM homomix" (4) for about 10 h, followed by drying and autoradiography (30 min to 4 h).

RESULTS

Purification and purity of viroids: Preparative electrophoresis on 5 % polyacrylamide gels was started with batches of approximately 2000 A_{260} units of "2 M NaCl soluble" RNA from viroid infected leaf tissue per 25 x 20 x 0.6 cm gel. Only this allowed the staining and location of the minute amount of viroid present in infected plant tissue as compared to the healthy control (2). This deliberate overloading of the first preparative gels causes considerable contamination of the viroid band by 5S RNA and tRNA due to partial retention during the electrophoretic separation. Therefore, two to three additional electrophoretic separations of the viroid isolated and concentrated from the pooled viroid bands of twenty to thirty gels were needed to achieve a final purity of at least 99 %. Criteria for the purity of viroids were a single infectious band in polyacrylamide gels, homo-

geneity upon analytical ultracentrifugation and the presence of only one RNA species in the electron microscope (2). Viroid purity was further substantiated by the oligonucleotide fingerprint patterns presented in Figure 1.

Evidence for the individuality of three viroid species: The three viroids CEV, PSTV and ChSV originate from their natural host plants citrus, potato and chrysanthemum, respectively, which are botanically unrelated. For mass propagation they were adapted to suitable experimental hosts which are Gynura aurantiaca for CEV, Lycopersicum esculentum (tomato) for PSTV and Cineraria hybrida for ChSV. It should be noted that no relationship exists between the natural and the experimental host in the case of CEV, whereas there is a rather close relationship between the solanaceous hosts of PSTV and the composite hosts of ChSV. Moreover, the experimental hosts for CEV and ChSV are also closely related composites. Despite certain host plant dependent differences the general symptom caused by all these viroids is leaf curling (epinasty) and stunting. These characteristic symptoms, especially evident after adaptation of "different" viroids to common experimental hosts, led to the assumption that viroids from different hosts are actually identical (18).

In order to clarify whether different viroids are identical or not we compared the oligonucleotide patterns of CEV, PSTV and ChSV obtained after RNAase cleavage, 3'dephosphorylation, and 5'^{32}P-phosphorylation of the oligonucleotide mixture, electrophoresis, homochromatography and autoradiography. Figure 1 shows the corresponding RNAase T_1 and RNAase A patterns. The following features deserve special attention:

(i) The number of oligonucleotides in these fingerprints and their intensity in the autoradiograms is consistent with a viroid size of approximately 320-380 nucleotides, as determined previously by other methods (2,3,11).

(ii) The fingerprints of the three viroids CEV-gyn, Figure 1a and 1d, PSTV-tom, Figure 1b and 1e, and ChSV-cin, Figure 1c and 1f show significant and characteristic differences in the RNAase T_1 as well as in the RNAase A patterns. This clearly demonstrates the individuality of the three different viroids investigated here.

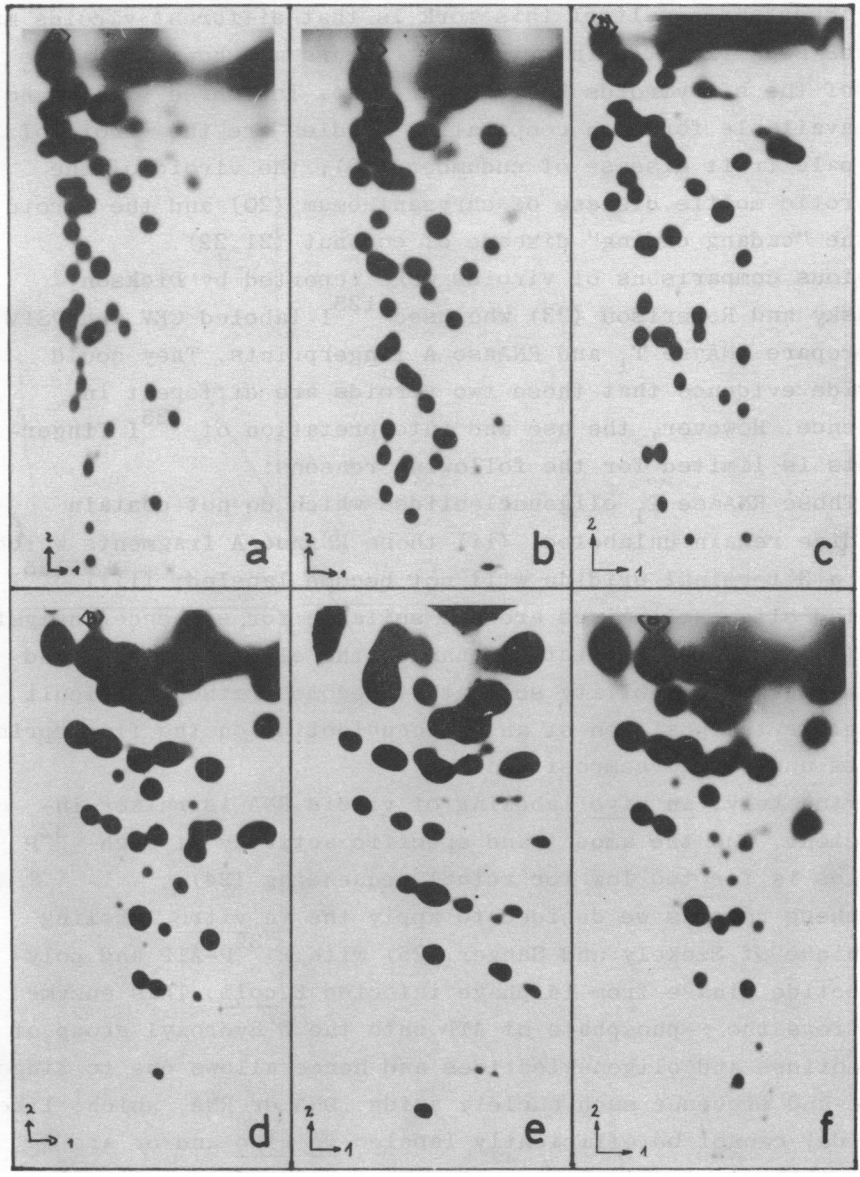

Fig. 1: Autoradiographs of 5′ ^{32}P labeled oligonucleotides. Oligonucleotides were obtained from RNAase T_1 digests of (a) CEV-gyn; (b) PSTV-tom; (c) ChSV-cin; and from RNAase A digests of (d) CEV-gyn; (e) PSTV-tom; and (f) ChSV-cin, respectively.

DISCUSSION

The important result of this work is that different viroids may differ significantly in primary structure as shown for three out of the six viroids known at present. The three viroids not yet available for such comparative studies are the viroid of the pale fruit disease of cucumber (19), the viroid of the chlorotic mottle disease of chrysanthemum (20) and the viroid of the "cadang cadang" disease of coconut (21,22).

Previous comparisons of viroids were reported by Dickson, Prensky and Robertson (23) who used ^{125}I labeled CEV and PSTV to prepare RNAase T_1 and RNAase A fingerprints. They could provide evidence that these two viroids are different in sequence. However, the use and interpretation of ^{125}I fingerprints is limited for the following reasons:

(i) Those RNAase T_1 oligonucleotides which do not contain cytidine remain unlabeled, (ii) those RNAase A fragments which have a 3′terminal uridine will not become labeled; (iii) ^{125}I labeled oligonucleotides are not suitable for sequence analysis and (iv) iodine substitution changes the electrophoretic and chromatographic mobility so that it becomes rather difficult to relate the position of an oligonucleotide on the fingerprint to its nucleotide composition.

Unfortunately, *in vivo* labeling of viroid RNA is rather inefficient, and the amount and specific activity of such ^{32}P viroids is far too low for actual sequencing (24).

For these reasons we decided to apply the *in vitro* labeling technique of Szekely and Sanger (25) with γ-^{32}P-ATP and polynucleotide kinase from T4 phage infected *E.coli*. This enzyme transfers the γ-phosphate of ATP onto the 5′hydroxyl group of nucleotides and oligonucleotides and hence allows one to fingerprint and sequence such nucleic acids, DNA or RNA, which, like viroids, cannot be efficiently labeled *in vivo* and/or are available only in minute amounts. The successful application of the above method has been demonstrated recently for tRNA (5,15,26,27,28). However, with this technique reliable results are only obtained when the RNA to be studied is of highest purity and when the polynucleotide kinase to be used is free from any traces of RNAase activity. It should be noted, that

the polynucleotide kinase fingerprints recently published for a so-called "fraction II" of PSTV from the solanaceous local lesion host Scopolia sinensis (29) are completely different from our patterns, and it is remarkable that they contain far less oligonucleotides than shown for PSTV in our study.

In conclusion, our findings confirm and extend the work of Dickson et al. (23) and support the concept of individual viroid species. Future work is aimed to include additional viroid isolates in our comparative studies and to establish their primary sequences.

ACKNOWLEDGEMENTS

We thank Mrs. K. Ramm, H. Muth, M. Bahr, I. Schlag and Mr. H. Thiele for their assistance during viroid propagation and purification.

This work was supported by the Deutsche Forschungsgemeinschaft through SFB 47 and personal grants.

REFERENCES

1 Diener, T.O. (1972), Adv. Virus Res. 17, 295-313.
2 Sänger, H.L., Klotz, G., Riesner, D., Gross, H.J. and Kleinschmidt, A.K. (1976), Proc.Natl.Acad.Sci. USA 73, 3852-3856.
3 Henco, K., Riesner, D., and Sänger, H.L. (1977), Nucleic Acids Res. 4, 177-194.
4 Jay, E., Bambara, R., Padmanabhan, R., and Wu, R. (1974), Nucleic Acids Res. 1, 331-353.
5 Gillum, A.M., Urquhart, N., Smith, M., and RajBhandary, U.L. (1975), Cell 6, 395-405.
6 Weathers, L.G., Greer, Jr., F.C. and Harjung, M.K. (1967), Plant Dis. Reptr. 51, 868-871.
7 Sänger, H.L. (1972), Advances in Biosci. 8, 103-116.
8 Diener, T.O. (1971), Virology 45, 411-428.
9 Hollings, M. and Stone, O.M. (1973), Ann.appl.Biol. 74, 333-348.
10 Diener, T.O., and Lawson, R.H. (1973), Virology 51, 94-101.
11 Sänger, H.L., and Riesner, D. (1977), J.Gen.Virol., in press.
12 Glynn, I.M., and Chappell, J.B. (1963), Biochem.J. 90, 147-149.
13 Richardson, C.C. (1965), Proc.Natl.Acad.Sci. USA 54, 158-165.
14 Panet, A., van de Sande, J.H., Loewen, P.C., Khorana, H.G., Raae, A.J., Lillehaug, J.R., and Kleppe, K. (1973), Biochemistry 12, 5045-5050.
15 Simsek, M., Ziegenmeyer, J., Heckman, J., and RajBhandary, U.L. (1973), Proc.Natl.Acad.Sci. USA 70, 1041-1045.
16 Sanger, F., Brownlee, G.G., and Barrell, B.G. (1965), J.Mol.Biol. 13, 373-398.
17 Southern, E.M. (1974), Anal. Biochem. 62, 317-318.
18 Semancik, J.S., Magnuson, D.S., and Weathers, L.G. (1973), Virology 52, 292-294.

19 van Dorst, H.J.M., and Peters, D. (1974), Neth.J.Pl.Path. 80, 85-96.
20 Romaine, C.P., and Horst, R.K. (1975), Virology 64, 86-94.
21 Randles, J.W. (1975), Phytopathology 65, 163-167.
22 Randles, J.W., Rillo, E.P., and Diener, T.O. (1976), Virology 74, 128-139.
23 Dickson, E., Prensky, W., and Robertson, H.D. (1975), Virology 68, 309-316.
24 Sänger, H.L. and Ramm, K. (1975), in: Modification of the information content of plant cells, pp. 229-252. Edited by R. Markham, D.R. Davies, D.A. Hopwood and R.W. Horne, Amsterdam: North Holland Publ.
25 Szekely, M., and Sanger, F. (1969), J.Mol.Biol. 43, 607-617.
26 Simsek, M., Petrissant, G., and RajBhandary, U.L. (1973), Proc.Natl.Acad.Sci. USA 70, 2600-2604.
27 Ghosh, K., Ghosh, H.P., Simsek, M., and RajBhandary, U.L. (1974), J.Biol.Chem. 249, 4720-4729.
28 Gillum, A.M., Roe, B.A., Anandaraj, M.P.J.S., and RajBhandary, U.L. (1975), Cell 6, 407-413.
29 Singh, R.P., Michniewicz, J.J., and Narang, S.A. (1975), Can.J.Biochem. 54, 600-608.